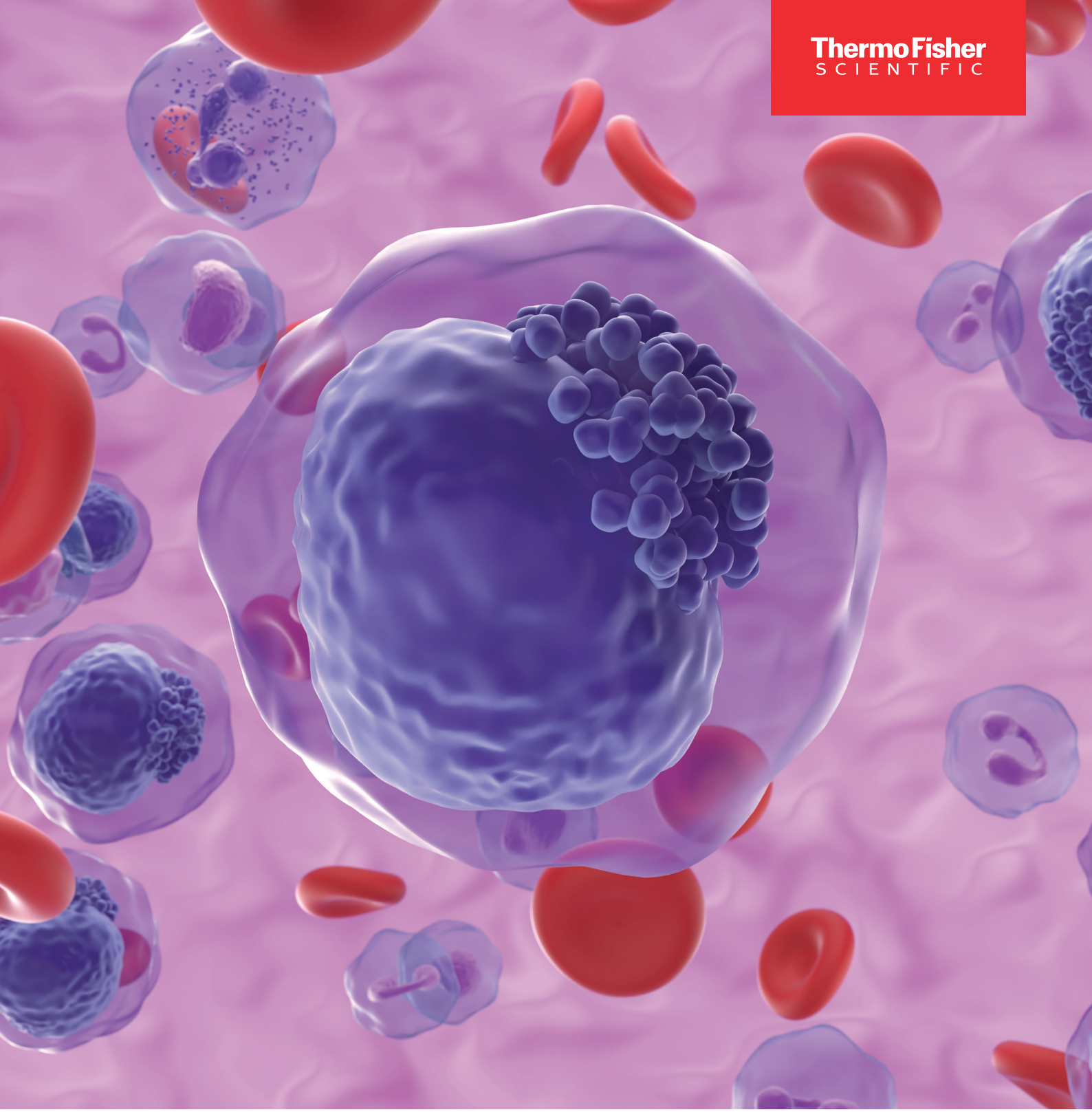

The growing importance of next-generation sequencing in myeloid molecular testing

How NGS is unlocking the power of precision oncology

Introduction

Advancing myeloid testing and precision medicine with NGS

The use of next-generation sequencing (NGS) for routine clinical care is rapidly accelerating.[1,2] This shift is due in part to advances in NGS technology, which have propelled the discovery of somatic mutations that play a pivotal role in hematological disorders and the associated development of targeted therapies.[2] These newly identified genetic alterations and molecular pathways provide valuable clinical insights across the continuum of care.[2]

Hematology has long been the proving ground for molecular testing.[1,2] Consequently, the clinical value of next-generation sequencing (NGS) is most apparent today in myeloid molecular testing.[1,2]

Traditional molecular testing involves iterative single-analyte testing modalities, complex workflows, and frequent outsourcing to referencing laboratories, all of which results in variable, often lengthy, turnaround times.[1,3] Associated delays in obtaining results can postpone diagnosis and treatment, negatively impact disease management, and be stressful for patients.[3]

With an ever-growing list of biomarkers, inherent genetic complexity, and the risk of rapid progression, myeloid malignancies challenge the current iterative testing paradigm and call for a streamlined testing approach that yields rapid results.[1]

Currently, NGS enables cost-effective, efficient in-house testing of multiple biomarkers on targeted gene panels via streamlined, automated workflows.[3] Results can be available within hours or days, depending on the platform.[3] With its demonstrated clinical utility in myeloid malignancies, NGS is transforming the testing paradigm and enabling better outcomes for patients.[1-3]

By leveraging the power of NGS to inform diagnosis, risk stratification, therapy selection, and monitoring in hemato-oncology, progressive pathologists are leading the way in precision medicine.[1,3]

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CHAPTER 1: Myeloid disorders

Major categories of myeloid malignancies

Myeloid malignancies arise from mutations in hematopoietic stem or progenitor cells.[4] These clonal disorders often exhibit high degrees of heterogeneity, complex karyotypes, and multiple categories of somatic mutations.[1,2,4]

The natural process of blood cell formation involves hematopoietic stem cell differentiation into myeloid and lymphoid cell lineages.[5] Hematopoietic disruptions in the myeloid lineage can lead to 3 major disease categories: acute myeloid leukemia (AML), myeloproliferative neoplasms (MPN), and myelodysplastic syndrome (MDS).[4,6] Examples of myeloid malignancies are as follows:

Acute Myeloid Leukemia (AML)

Myeloproliferative Neoplasms (MPN)

Myelodysplastic Syndromes (MDS)

Chronic Myeloid Leukemia (CML)

Polycythemia Vera (PV)

Essential Thrombocythemia (ET)

Primary Myelofibrosis (PMF)

The challenges of molecular testing in hemato-oncology

Due to their genetic complexity and high degree of heterogeneity, myeloid malignancies can be challenging for molecular analysis.[1] Since some myeloid disorders like AML can progress rapidly, the European LeukemiaNet (ELN) recommends that molecular testing for some biomarkers (eg, *NPM1* and *FLT3*) be available on a rapid-turnaround basis, within 3 to 5 days for AML.[1,7]

Over the last decade, whole-genome and whole-exome sequencing studies have revealed a number of recurrent mutations associated with these disorders. As the list of biomarkers expands so do the molecular testing algorithms, making it impractical—if not implausible—for laboratories to keep pace using single-gene testing methods.[1,8]

NGS helps overcome these challenges by empowering laboratories to perform a multi-gene assessment with a single test and enabling rapid results.[1,8]

Ultimately, real progress depends on the ability of all molecular testing laboratories (institutional, hospital, and community) to keep pace and to deliver the answers that clinicians need at each stage of the care continuum. Realizing the promise of precision oncology and its potential for better outcomes in hematology-oncology and beyond entails a new molecular testing paradigm—one where in-house, streamlined biomarker profiling with rapid results is not only possible, but ubiquitous.

CHAPTER 2: The necessity for in-house NGS

Clinician's perspective

From a clinician's perspective, the biggest challenges of serial single-gene testing and NGS outsourcing are the variable turnaround times and associated delays in patient care.[8,9] A complete mutational profile can take weeks to deliver. For acute and aggressive hematological malignancies, clinicians often require tests with rapid turnaround time (TAT) to enable timely identification of disease subtype and treatment plan development.[1,7]

Laboratory's perspective

From the laboratory's perspective, the limited scope of single-gene assays can be a significant drawback as each test requires time, resources, and separate samples. Additional disadvantages of Sanger sequencing, for example, are its low sensitivity and inability to detect subpopulations reliably or work efficiently with limited sample quantities.[8] As such, sequential single-gene testing and NGS outsourcing can take a considerable toll on a laboratory's labor and overhead costs.[8]

Assay development perspective

From an assay development perspective, aside from being highly resource-intensive, it's not feasible that laboratories can keep up with the rapid pace of biomarker discovery.[8]

Consequently, the complexities, costs (tangible and intangible), and impracticalities of performing and developing multiple single-gene assays are beginning to undermine their benefits.[8]

Optimizing molecular testing with in-house NGS

In-house NGS testing overcomes these challenges by enabling laboratories to profile all relevant genetic aberrations simultaneously in a massively parallel fashion. NGS generally provides higher sensitivity, larger scale, and the ability to detect novel aberrations compared to traditional methods (Table 1).[10]

In fact, parallel sequencing of targets with NGS can allow a laboratory to identify all relevant point mutations, insertions, deletions, translocations, and copy number variations associated with a given disorder.[8] With improvements in NGS technology, some platforms can even allow simultaneous assessment of DNA and RNA targets.[8,11] The primary clinical benefit of in-house NGS testing is that laboratories can produce a complete mutational profile in as fast as one day with the latest technologies.

Table 1: Comparison of various molecular testing methods.

Test	Application	Benefits	Limitations
Sanger Sequencing	Interrogation of a single gene – must have a known gene region of interest	• Cost effective • Quick and simple workflow	• Sensitivity (only down to ~20% allele fraction) • Low discovery power • Not cost-efficient for large regions of DNA • Low scalability
Quantitative real-time PCR (qPCR)	Analysis of specific gene variants at specific locations – researcher must know exactly what and where to interrogate	• High sensitivity • Quick and simple workflow • Easily acquired equipment	• Limited to only a few gene variants or fusions • No discovery power
Targeted NGS	Multiple gene variants across targeted areas of the genome (tens to hundreds of genes)	• High sensitivity • High discovery power (comprehensive genomic coverage) • Greater variant resolution • More data from smaller amounts of DNA • Higher throughput	• Requires data-handling workflow or bioinformatics • Smaller number of samples may be less cost-effective

Takeaway: Sanger sequencing and qPCR are best leveraged to interrogate small regions of DNA.[10] To screen multiple samples and detect variant types across specific areas of the genome, targeted NGS is more efficient and cost-effective.[8]

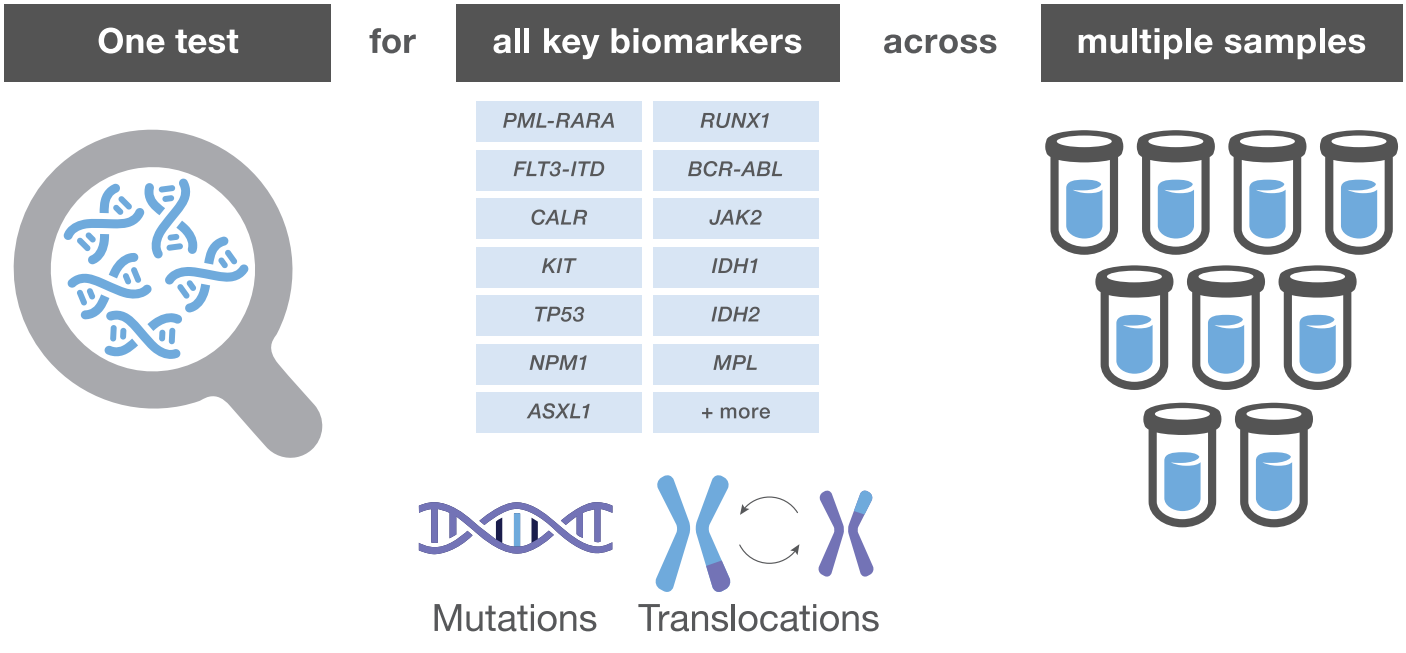

Figure 1: NGS allows simultaneous profiling of key biomarkers across multiple samples with a single test.

Simultaneous detection of multiple types of variants

Given the substantial phenotypic and genetic heterogeneity of myeloid disorders and the continually increasing number of alterations that need to be analyzed, many more laboratories are integrating NGS into their diagnostic algorithms.[1,3,9]

By providing a complete analysis of genetic alterations, including single-nucleotide variants, translocations, and small insertions and deletions, NGS myeloid panels can enable enhanced differentiated disease classification, risk stratification, and improved therapeutic decisions.[1,3,9]

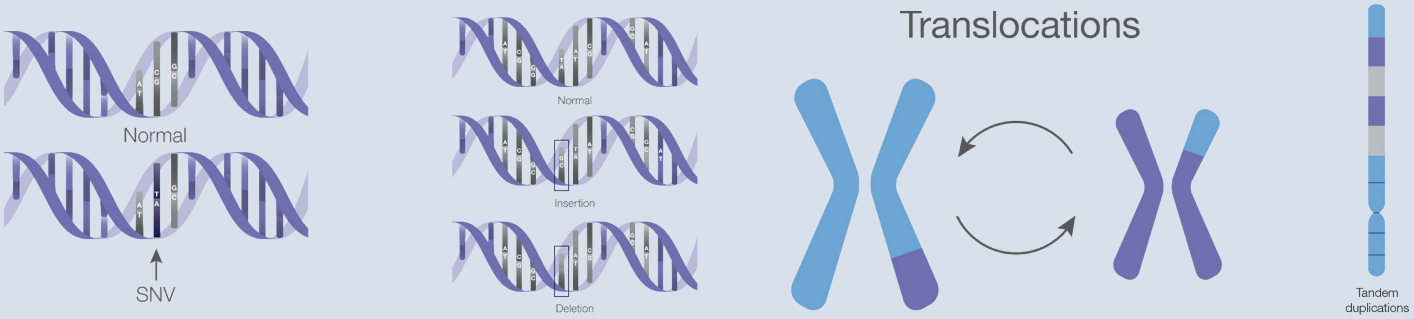

Figure 2: Examples of multiple variant types that can be detected with NGS.

Dr. Bekim Sadokovic

Western University

Expert opinion

The classic approach to molecular hematology testing involves targeted single-analyte testing after initial pre-screening by other diagnostic modalities including flow cytometry, anatomic pathology, and clinical cytogenetics. This approach presents several limitations:

- Every patient specimen must be stabilized, DNA and RNA extracted and stored regardless of whether molecular testing will be performed. Subsequently, a specific targeted molecular assay may be activated for a subset of patient.
- Commonly, the assay targets (specific genes) chosen are a "best guess" based on preliminary findings from the initial pre-screening.
- Targeted testing may exhaust available scarce tissue specimens such as bone marrow rendering additional targeted assays impossible.
- Turnaround times are negatively impacted due to delays in initiating targeted molecular testing.
- Furthermore, there are significant personnel requirements to manage the pre-screening tests and to determine which samples require targeted molecular assessment and by which specific molecular assays.

"Given that whole genome or exome sequencing has revealed a wide but limited-range of high-frequency mutations associated with hematologic malignancies such as myeloid neoplasms, including DNA sequence mutations and gene fusions, a DNA/RNA-based NGS panel provides an ideal alternative approach for comprehensive testing of patients as an initial, tier-1 screen in place of targeted triage, rather than as a secondary (tier-2) assay reflexive to other tests."[4]

CHAPTER 3: NGS methods

Whole-genome (WGS)

WGS identifies every DNA base across the entire genome (coding, noncoding, mtDNA) and detects all variant types (SNVs, indels, SVs, CNVs).[12] With its massive data set, WGS entails substantial costs, extended time to results (weeks), and complex data interpretation, making it a method better suited for research and discovery rather than routine clinical testing.[3,12]

Whole-exome sequencing (WES)

Targeting the protein-coding regions of the genome, WES detects coding variants (SNVs, indels).[12] Due to the cost, length of time to results (weeks), and the complexity of data interpretation, WES is better suited for research and discovery than routine clinical testing.[3,12]

Whole-transcriptome (WTS) sequencing

WTS identifies genome-wide differential RNA expression (coding and noncoding). By evaluating altered genetic variants and the continuously changing cellular transcriptome, WTS provides valuable information about the cells and transcriptional networks.[13] It is commonly used for discovery and gene-expression analysis.[13]

Targeted sequencing

Targeted-sequencing panels profile a select set of genes that are curated for a specific application or disease.[13] Featuring actionable biomarkers and a smaller data set, these panels are commonly used in clinical applications to help inform the diagnosis, treatment, and monitoring of individual patient disease states.[3]

Targeted NGS panels help inform clinical management of myeloid disorders

When it comes to acute hematological malignancies, early diagnosis and personalized disease management present substantial challenges, as does the reliable and timely detection of measurable residual disease (MRD) —essential to guiding therapy choices, monitoring treatment response, and detecting relapse.[3] Genomic biomarkers are the key to unlocking this valuable clinical information.[1,3,7]

While the value of WGS and WES in studying hematological malignancies is recognized, applying these methods for routine clinical testing is impractical due to costs, long turnaround time to results (weeks), and the complexities of data storage, analysis, and interpretation.[3]

With their actionable biomarkers, quick turnaround times, and lower cost compared to WES, targeted NGS gene panels are best suited for routine clinical applications and especially ideal for identifying mutations and translocations in specific genes.[1,3,8] Some advanced NGS platforms deliver robust integrated analysis and reporting tools, empowering clinicians to facilitate patient selection and clinical trial inclusion.[14,15]

Relying on the high sensitivity of NGS, clinicians can utilize it to monitor multiple mutations over time to better track a patient's evolving mutational landscape.[7,16] NGS promises to advance precision medicine by identifying actionable biomarkers and individual disease mutations and matching patients to clinical trials, all ultimately contributing to the development of novel, targeted therapies, tailored disease management, and better outcomes.[1,3,14,15]

Expert opinion: NGS testing considerations in molecular

During a recent presentation, Craig Mackinnon, MD, PhD, director of Genomic Diagnostics at the University of Alabama, discussed the key considerations for setting up a NGS panel in a molecular oncology research laboratory. Basic requirements included the capacity to target genes of interest and detect a wide range of variants, including single nucleotide variants (SNVs), small indels, and fusions, all while accommodating multiple specimen types of varying quality and input amounts.

Dr. Mackinnon concluded that **automation, hands-on time, turnaround time, and the associated potential for cost savings** should all be part of the evaluation.

Consolidates multiple tests into 1 panel	✓
Detects a wide range of targets	✓
Accommodates a variety of specimen types (sample quality, input amount)	✓
Features an automated workflow with minimal hands-on time	✓
Delivers a rapid turnaround time	✓
Incorporates automated bioinformatics analysis and reporting	✓
Includes variant interpretation software	✓
Offers the potential for cost savings	✓

Results are needed faster for more markers

The most recent ELN guidelines now recommend an increased number of mutations including gene fusions have testing results available in **3 to 5 days.[7] Single gene testing is increasingly replaced by gene panel diagnostics** given the number of biomarkers that now require molecular analysis. [7]

Biomarkers with testing results recommend within 3 to 5 days by ELN

Table 2: Examples of biomarkers that are recommended to be tested within 3 to 5 days.

Alteration	Associated disease	Significance
PML::RARA	APL	Diagnostic indication for APL
FLT3-ITD	AML	Targetable mutation
FL3-TKD	AML	Targetable mutation
IDH1	AML	Targetable mutation
IDH2	AML	Targetable mutation
CBFB::MYH11	AML	Diagnostic fusion
RUNX1::RUNX1T1	AML	Diagnostic fusion
KMT2A rearrangements	AML	Diagnostic fusion
BCR::ABL1	AML/CML	Diagnostic fusion
NPM1	AML	Risk Assessment
Other fusions	AML	Diagnostic fusions

CHAPTER 4: The value of NGS across the care continuum

A giant step forward for disease management and precision oncology

Advances in NGS have made simultaneous assessment of multiple target genes possible in daily laboratory analysis.[1,3] Targeted NGS panels designed explicitly for myeloid neoplasms can inform patient-management decisions across the entire continuum of care—from streamlining diagnosis, identifying disease subtypes, facilitating treatment decisions, assessing risk stratification, and providing prognostic insights to improve monitoring and measurable residual disease (MRD) detection.[1,3,9]

As in-house NGS testing becomes ubiquitous at a community hospital-laboratory level, more patients will be able to benefit from these important advancements in precision oncology.

Figure 3: The value of NGS across the care continuum.

Streamlined diagnosis

Historically, most community-based laboratories have utilized traditional single-analyte molecular testing methods, outsourcing to reference laboratories as needed to augment their in-house capabilities.[17,18]

This strategy presents a challenge when multiple biomarkers need to be tested, as is the case for myeloproliferative neoplasms (MPNs). Sequential iterative testing can contribute to substantial delays in diagnosis and treatment.[1,3,8,9] These delays can be time and cost-intensive for laboratories, frustrating for clinicians, and angst-provoking for patients.[1,3,8,9]

Today, multi-gene NGS panels enable laboratories to analyze all relevant genes using a single test that yields results within days.[3] With NGS technology delivering expedited results, clinicians will have the information they need to optimize disease management sooner, and patients will have peace of mind.[1-3]

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Rapid turnaround time

Rapid assessment of genetic biomarkers in myeloid malignancies is vital for early diagnosis, first-line therapy choices, and ongoing clinical disease management.[3,6] This includes evaluation of therapy response and relapse/measurable residual disease (MRD) detection.[3,6] In particular, acute diseases like AML require rapid test results for key diagnostic and risk stratification markers (ie, *PML::RARA* gene fusion, *FLT3* or *IDH1/2*, and *NPM1*).[3,7] The 2022 ENL guidelines recommend that results for *FLT3, NPM1*, and other key targets, to be available within 3 to 5 days.[7]

"There are several protocols, including extensive amounts of genetics and immunophenotyping, which are required to establish the appropriate approach to treat patients. The sooner we can get results and deliver treatment, the better the outcomes."

Advances in AML risk-stratification guidelines and targeted therapies have made it increasingly important for clinicians to consider a wide range of genetic biomarkers to guide front-line patient-care decisions.[3,6,7] NGS helps to address this need by rapidly providing results for a broad number of genetic targets.[3] The latest instruments enable a laboratory to sequence a patient sample in about 24 hours so clinicians can have the results in days.[3]

Disease subclassification: AML

Many myeloid malignancies are classified by the presence of specific genetic mutations.[1,3] While an initial diagnosis can be achieved with histopathological and immunophenotyping tests, disease subtypes are usually determined through karyotyping and genetic analysis.[1] There are approximately a dozen genomic alterations and an average of 3 driver mutations identified per AML sample.[19]

NGS offers critical advantages in AML disease subclassification as all relevant mutations can be identified in a single test.[19] Some of the latest NGS platforms deliver results within hours or days, so these mutational insights can be evaluated in conjunction with the histopathological or immunophenotyping results for a more complete picture of the disease pathology.[3]

Indeed, the inclusion of certain mutations in the key treatment guidelines provides strong evidence for how mutational testing is influencing clinical disease management.[6,7,20] Genetic profiling and disease subclassification is expected to become increasingly important in daily clinical practice.[21]

Risk stratification: AML

Genetic abnormalities are powerful prognostic factors that can help inform therapy selection.[7,22] Some genetic mutations can also indicate the suitability for stem cell transplantation (allo HCT) in AML patients.[7,22] Results from conventional cytogenetics and NGS mutation screenings are routinely used in clinical practice for such purposes.[8]

Changes to medical guidelines and classification criteria for AML have added pressure on laboratories to increase molecular testing for more genetic biomarkers and deliver results in just days.[7] Traditional approaches involving multiple single-analyte tests may be increasingly perceived as less sustainable and less efficient than NGS in providing comprehensive molecular analysis across several genes.

2022 ELN risk classification by genetics at initial diagnosis

Risk category	Genetic abnormality
Favorable	• *RUNX1::RUNX1T1* • *CBFB::MYH11* • Mutated *NPM1* without *FLT3-ITD* • bZIP in-frame mutated *CEBPA*
Intermediate	• Mutated *NPM1* with *FLT3-ITD* • Wild-type *NPM1* with *FLT3-ITD* (without adverse-risk genetic lesions) • *MLLT3::KMT2A* • Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	• *DEK::NUP214* • *KMT2A*-rearranged • *BCR::ABL1* • *KAT6A::CREBBP* • *GATA2, MECOM(EVI1)* • *MECOM(EVI1)*-rearranged • –5 or del(5q); –7; –17/abn(17p) • Complex karyotype, monosomal karyotype • Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2*, *STAG2, U2AF1, and/or ZRSR2* • Mutated *TP53*

Through the identification of SNVs, insertions, deletions and rearrangement, NGS offers distinct advantages for risk stratification.[23] Given the sheer number of genetic abnormalities that must be evaluated, a multi-gene NGS panel can consolidate many, if not all of the individual single-gene tests that would otherwise be required.[3,8]

Currently, NGS is the most cost-effective method of risk assessment for AML.[3] By utilizing in-house NGS, laboratories could potentially accelerate time to results while simultaneously lowering costs and reducing the strain on its resources.[1,3,8]

Therapy selection

Since the 1970s, the standard of care for AML has been cytotoxic chemotherapy, and long-term overall survival has remained relatively poor.[24] Progress in the development of mutation-targeted therapies in recent years is transforming the AML treatment paradigm.[24,25] Finally, there are more options for patients, and with them, the promise of better clinical outcomes.[24]

NGS has helped identify many significant genetic alterations, leading to the development of therapeutics that target specific gene mutations.[24,26] In 2017, midostaurin became the first tyrosine kinase inhibitor (TKI) approved for *FLT3*-mutated AML; many more targeted therapies are emerging, including the *IDH2* inhibitor, enasidenib for relapsed/refractory (R/R) AML.[24] While further research is needed to understand all the genomic complexities at play, it is apparent that these new targeted therapies are raising the standard of care for AML and improving overall survival and quality of life.[24]

With its comprehensive genetic profile of the disease, including targetable mutation and other genetic abnormalities, NGS can offer tremendous clinical insights into AML, from risk stratification and therapy selection to disease monitoring.[24]

Explosion of new AML therapy options in recent years[24,26]

FDA-approved agents for AML

1973
7+3 induction chemotherapy
AML

1990
Idarubicin
AML

2000
Arsenic trioxide
Target: PML-RARA
APL

2017
Midostaurin
Target: FLT3
Newly diagnosed *FLT3*-mutated AML

CPX-351
t-AML or AML-MRC

Gemtuzumab ozogamicin
Newly diagnosed and R/R *CD33*-positive AML

2018
Gilteritinib
Target: FLT3
R/R *FLT3*-mutated AML

Enasidenib
Target: IDH2
R/R *IDH2*-mutated AML

Glasdegib
Target: Hedgehog
Newly diagnosed AML (age >75)*

Venetoclax
Target: BCL2
Newly diagnosed AML (age >75)*

2019
Ivosidenib
Target: IDH1
Newly diagnosed *IDH1*-mutated AML (age >75)*

2020
Oral azacitidine
AML (in first remission)

2022
Olutasidenib
Relapsed or Refactory *IDH1*-mutated AML

2023
Quizartinib
Newly diagnosed *FLT3*-ITD mutated AML

2024
Revumenib
AML with *KMT2A* rearrangements, in relapse/refractory

R/R = Relapse/Refractory

* Patients >75 years old or who have comorbidities that preclude the use of intensive induction chemotherapy

Recurrence monitoring

Recurrence monitoring and measurable residual disease (MRD) detection are critical for hematological disorders management.[1,3,9] While our improved understanding of the molecular landscape of AML has resulted in more informed and targeted first-line treatments, the threat of disease relapse is always looming.[24] Studies have shown that negative MRD (assessed by molecular techniques or immunophenotyping) may be a better predictor of survival as it's associated with a lower risk of relapse.[27]

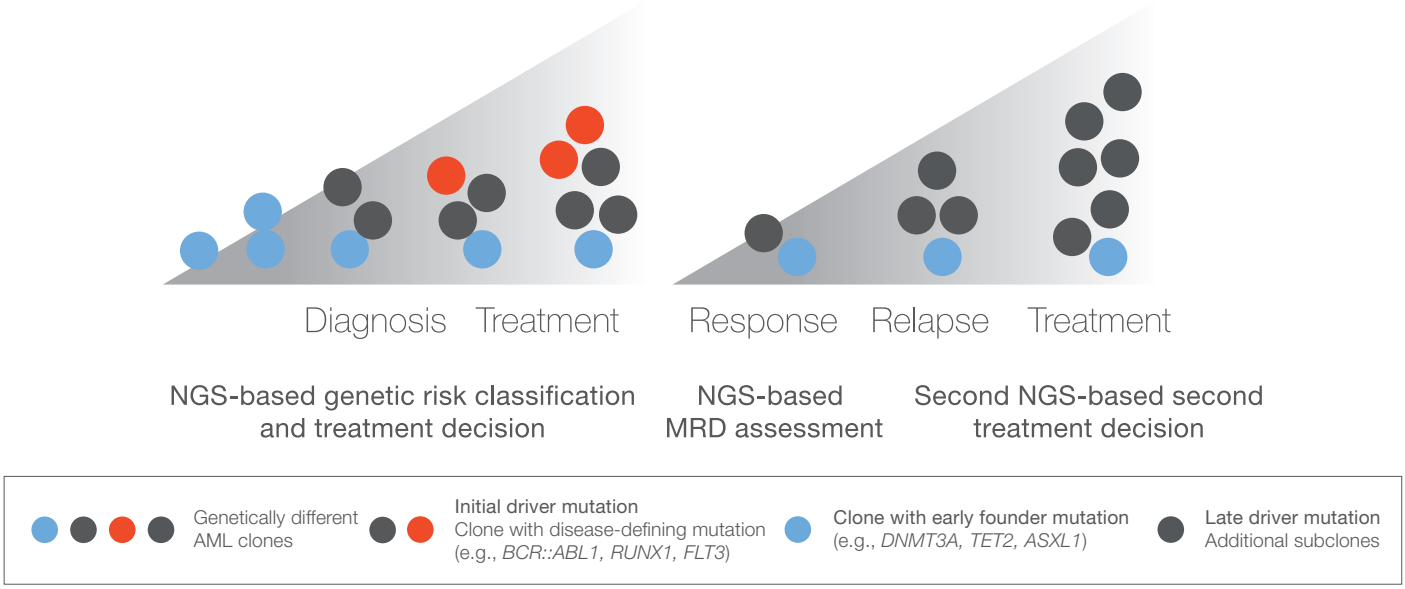

Figure 4: NGS identification of clonal and subclonal mutations in AML provides insights into prognosis, treatment, and response.[7,15]

Molecular monitoring methods: MFC, qPCR, and NGS

Molecular techniques, such as multiparameter flow cytometry (MFC) and qPCR, are commonly used to measure and monitor the disease burden of myeloid disorders.[27,28,29]

MFC has been the gold standard for MRD detection, but it is operator- and center-dependent without a standardized method of enumerating flow-based MRD in AML.[29] Most notably, MFC may lack sensitivity for detecting residual disease.[1]

qPCR assays are highly sensitive but have limited utility in monitoring AML because they only identify common mutations and translocations, which are present in just 15% to 25% of AML cases and even fewer MDS cases.[30,31]

qPCR is challenged by clonal evolution post-chemotherapy and the significant time, cost, and resources required to design patient-specific primers, making it impractical for AML monitoring.[1,3,30]

Benefits of NGS for MRD in AML

Given AML's combinatorial complexity and mutational combinations (3 to 5 driver mutations in more than 250 genes), NGS can detect the persistence of disease-specific variants to inform treatment and prognosis and identify patients at high risk for early relapse.[1,3,25]

Benefits of NGS:

- Monitors multiple mutations simultaneously: SNVs, insertions, deletions, fusions.[1,3,32]
- Sensitive enough to track small clonal population and evolution.[1,3,32]
- Features the convenience of a universal assay, eliminating the need for allele-specific primers.[32]
- Is a cost-effective screening method.[3,8]

CHAPTER 5: Outlook & key considerations

NGS: Advancing precision oncology

Advances in NGS workflow automation and integrated reporting have empowered clinicians to perform simultaneous assessments of multiple target genes in routine laboratory analysis.[1,3,8] An ever-growing list of biomarkers and their inherent genetic complexity have made myeloid malignancies the proving ground for this new testing paradigm.[1,2]

The value of integrating NGS into daily clinical practice for rapid, streamlined analysis and utility across the care continuum is no longer in question.[1,3] Assessment of myeloid neoplasms with targeted NGS panels is demonstrated to improve diagnosis, assist therapeutic decisions, inform prognosis, and better detect measurable residual disease.[1] Adoption of in-house NGS brings advanced molecular diagnosis and precision oncology to more patients and can help raise the standard of care.[1-3]

Realizing the promise of precision oncology

Historically, cancer treatment was limited to a one-size-fits-all model, where therapies were selected based on the cancer type (site) alone. Today, NGS has advanced the understanding of cancer biology so many therapy decisions can be based on specific cancer biomarkers, and genetic alterations in an individual's cancer genome.[1-3]

Empowered with a molecular profile of a patient's cancer, clinicians can tailor treatments to optimize outcomes and prescribe therapeutics that directly target biological pathways while avoiding sub-optimal therapies.[1-3]

Imagine every oncology patient receiving treatment individualized to their specific cancer genome. What was once a vision for improved cancer care is finally becoming a reality—a world where molecular can help transform clinical care, improve outcomes, and enhance the quality of life for patients.

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